IGF-I signalling in bone growth

IGF-I SIGNALLING IN BONE GROWTH: INHIBITORY ACTIONS OF DEXAMETHASONE AND IL-1β

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ABSTRACT

Objective

To determine if glucocorticoids and proinflammatory cytokines inhibit bone growth through a common mechanism involving impaired IGF-I signalling.

Design

IGF-I (100ng/ml), dexamethasone (dex) (10^{-6}M) and IL-1ß (10ng/ml) with inhibitors of the PI3K (LY294002) and Erk1/2 (PD98059 and UO126) IGF-I pathways (all 10μM) were studied using the ATDC5 chondrocyte cell line and murine fetal metatarsal cultures.

Results

IGF-I stimulated ATDC5 chondrocyte proliferation (322%; $p<0.001$ versus control). Addition of PD or LY individually to IGF-I supplemented ATDC5 cultures partially reduced proliferation by 32% ($P<0.001$), and 66% ($P<0.001$) respectively. PD and LY in combination blocked all IGF-I stimulated ATDC5 proliferation. LY significantly reversed IGF-I stimulatory effects on metatarsal growth ($P<0.001$), whereas PD and UO treatment had no effect. IGF-I induced ATDC5 proliferation was further decreased when Dex (24%; $P<0.01$) or IL-1ß (33%; $P<0.001$) were added to PD but not LY cultures. Metatarsal growth inhibition by LY was unaltered by Dex or IL-1ß addition.

Conclusions

Both the PI3K and Erk1/2 pathways contributed independently to IGF-I mediated ATDC5 proliferation. However in metatarsal cultures, the Erk1/2 pathway was not required for IGF-I stimulated growth. Dex and IL-1ß may primarily inhibit IGF-I induced bone growth through the PI3K pathway.

INTRODUCTION

Poor linear growth that is often observed in chronic inflammatory conditions in childhood is attributed to a number of factors including exposure to glucocorticoids (GC), malnutrition, increased catabolism and functional defects in the GH/IGF-I axis [1]. Systemic concentrations of proinflammatory cytokines such as interleukin-1-beta (IL-1β), tumour necrosis factor-alpha (TNFα) and interleukin-6 (IL-6) are often raised in these conditions, and there is increasing evidence that these cytokines may themselves affect longitudinal growth [2]. IL-6 has been reported to modulate the systemic GH/IGF-I axis [3], and TNFα and IL-1β may directly inhibit growth plate chondrocyte dynamics [4, 5]. Chronic inflammatory conditions may be associated with GC exposure to agents such as prednisolone or dexamethasone (Dex). The impaired growth and skeletal development that is often observed following prolonged exposure to these agents [6] can also be partly attributed to their direct effects on growth plate chondrogenesis [7].

The cellular mechanism by which inflammatory cytokines and GCs inhibit bone growth remains elusive. It is also unclear if a common mechanism exists that may explain these inhibitory actions. One such mechanism may be the impairment of the insulin-like growth factor (IGF) axis. IGF-I can completely reverse the growth-inhibitory effect of Dex [7] and can partly ameliorate the growth-inhibitory effect of pro-inflammatory cytokines [4].

The IGF-I signalling pathway has a central function in modulating endochondral bone growth and regulates a number of key chondrocyte physiological processes such as cellular proliferation, differentiation and survival [8]. After ligand stimulation of the IGF-

I receptor tyrosine kinase, a family of endogenous substrates including insulin receptor substrates are phosphorylated. These proteins serve as docking sites for phosphoinositide 3-kinase (PI3K), whose activation can cause the recruitment of down stream signalling molecules such as Akt, and p42/p44 mitogen activated protein kinase (Erk 1/2) [9]. Although it is recognised that the relative contributions of each pathway to the control of mitogenesis and cell survival by IGF-I is cell type specific [10] recent data has indicated that both the PI3K and Erk 1/2 intracellular pathways mediate the IGF-I response in growth plate chondrocytes [11].

The aims of the present study were to identify the IGF-I signalling pathways responsible for chondrocyte proliferation and bone growth, and to determine if a common pathway is inhibited by dexamethasone and IL-1ß.

MATERIALS AND METHODS

Reagents

Dexamethasone (Dex) (Sigma, Poole, Dorset, UK) and IL-1ß (Autogen Bioclear, Calne, Wiltshire, UK) were added at final concentrations of 10^{-6}M and 10 ng/ml respectively. Dose response curves for inhibitors of the IGF-I pathway were used to determine the lowest concentration that inhibited IGF-I induced proliferation (data not shown). Inhibitors of the PI3K (LY294002; Sigma) and Erk1/2 (PD98059; Sigma and UO126; Cell Signalling Technology, Beverly, MA) IGF-I pathways were all added at final concentrations of 10μM, as used by others [12-14]. The diluent for LY, PD and UO was dimethyl sulfoxide (DMSO) (final concentration 0.3%). All control cultures received 0.3% DMSO only.

Chondrocyte cell culture

The ATDC5 chondrocyte cell line was sourced from the RIKEN cell bank (Ibaraki, Japan) and maintained as described by Atsumi *et al.* [15]. Cells were cultured as described previously [5]. In all experiments, reagents were added to chondrocyte cultures on Day 7. ATDC5 cells were deprived of serum and insulin/transferrin/selenium (ITS) for 18h before the initiation of treatments in the presence of IGF-I (100ng/ml) (Bachem (UK) Ltd., St. Helens, Merseyside, UK).

Chondrocyte proliferation

The rate of chondrocyte proliferation was assessed in cells cultured in 48 well plates over a 24 hour period starting on Day 7. On Day 8, the chondrocytes were incubated with 0.2μCi/ml [^{3}H]thymidine (37MBq/ml; Amersham Pharmacia Biotech, Bucks, UK) for the

last 2 hours of the culture period. The amount of radioactivity incorporated into trichloroacetic acid-insoluble precipitates was measured.

Organ culture

The middle three metatarsals were aseptically dissected from 19-day-old embryonic Swiss mice. Bones were cultured and morphometric analysis undertaken as described previously [7]. The experimental protocol was approved by Roslin Institute's Animal Users Committee and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals.

Statistical analysis

All experiments were performed at least twice. General Linear Model analysis was used to assess the data. All data are expressed as the mean +/- S.E.M. of six observations within each experiment. Statistical analysis was performed using Minitab 14. $P<0.05$ was considered to be significant.

RESULTS

IGF-I stimulated ATDC5 proliferation (Fig. 1A; 322%; $p<0.001$ versus control). The addition of PD (inhibitor of the Erk1/2 pathway) or LY (inhibitor of the PI3K pathway) to IGF-I supplemented cultures reduced proliferation by 32% ($P<0.001$), and 66% ($P<0.001$) respectively (Fig. 1A). Only the combination of PD and LY blocked all IGF-I stimulation of proliferation. Comparable results are also shown in Figure 1B. This data therefore indicates that both the Erk1/2 and PI3K intracellular pathways contribute independently to IGF-I mediated ATDC5 proliferation.

In the metatarsal model, the Erk 1/2 pathway was not required for IGF-I stimulated growth. LY reversed the majority of the IGF-I stimulatory effects (Fig. 2A; $P<0.001$) on bone growth, whereas PD and UO treatment had no effect (Fig. 2A and B).

IGF-I induced ATDC5 proliferation was decreased by both Dex (Fig. 1A; 22%; $P<0.01$) and IL-1ß (Fig. 1B; 40%; $P<0.001$). Proliferation was further decreased when Dex (Fig. 1A; 24%; $P<0.01$) and IL-1ß (Fig. 1B; 33%; $P<0.001$) were added to PD cultures but not to LY cultures. This suggests that Dex and IL-1ß primarily affect the PI3K pathway, and not the Erk1/2 pathway, which may explain the observed inhibition of IGF-I induced metatarsal growth in the presence of Dex (Fig. 3A; 17%; $P<0.05$) and IL-1ß (Fig. 3B; 23%; $P<0.001$). Metatarsal growth was inhibited by LY, which was not compounded by the addition of Dex or IL-1ß, again suggesting that Dex and IL-1ß primarily affect the PI3K signalling pathway.

DISCUSSION

The insulin-like growth factor–I (IGF-I) signalling pathway is unequivocally a major autocrine/paracrine regulator of bone growth. The results of this study showed that both the PI-3K and Erk 1/2 intracellular signalling pathways are responsible for mediating the ATDC5 chondrocyte proliferative effects of IGF-I. Using inhibitors with recognised specificity to PI-3K [16] and MEK [17], the inhibition of each separate pathway resulted in a partial reduction of IGF-I stimulated ATDC5 proliferation. Total inhibition of IGF-I stimulated ATDC5 proliferation was only observed in the presence of both inhibitors, although their effects on other endogenous activators of signalling pathways are unknown in this model. This observation that both the Erk 1/2 and PI-3K intracellular pathways contribute independently to IGF-I mediated chondrocyte proliferation is in agreement with studies on human intestinal smooth muscle cells [18]. However, it is interesting to note that whilst both the PI-3K and Erk 1/2 intracellular signalling pathways mediated the proliferative effects of IGF-I in the ATDC5 chondrocyte cell culture model, IGF-1 induced bone growth, as assessed in the fetal metatarsal model, occurred solely through the PI-3K pathway. The metatarsal model is a more physiological model of bone growth than the teratocarcinoma derived ATDC5 cell line, and is likely to more accurately represent the intracellular signalling pathways present. Therefore, the pro-proliferative effects of IGF-I on the growth plate may be mediated entirely through the PI-3K pathway.

The cellular mechanisms through which proinflammatory cytokines and GCs modulate bone growth have yet to be elucidated, however a common mechanism, such as the impairment of the IGF-I axis, may be involved. This is the first study to describe the effects of proinflammatory cytokines and GCs on both the PI-3K and Erk 1/2 intracellular

IGF-I signalling pathways in growth plate chondrocytes. The data from both the ATDC5 cell culture studies, and the metatarsal organ cultures suggest that Dex and IL-1β primarily inhibit IGF-I signalling through the PI3K signalling pathway. This is in agreement with previous studies in ATDC5 cells, in which Dex inhibited insulin-induced chondrogenesis by preventing PI3K-Akt signalling [19]. In other cell systems Dex has been shown to inhibit IGF-I action by inhibiting PI-3K activity in myoblasts. [20]. Additionally, studies in breast carcinoma cells have shown that IL-1β inhibits cell proliferation through suppression of Akt activation [21].

A possible contributing mechanism of action in this study could also be proinflammatory cytokine and GC induced modulatation of bone growth through altered endogenous IGF-I production. Studies in a rat osteoblast culture model have shown that Dex causes a dose-dependant inhibition of IGF-I synthesis [22]. Furthermore, IL-1β is associated with the inflammatory response, which includes the induction of cyclooxygenases, which are responsible for prostaglandin E_2 synthesis. Prostaglandin E_2 has significant effects on the expression of IGF-I, IGF-I receptors and IGF-I binding proteins [23, 24], which potentially complicates the interpretation of our data.

The actions of IL-1β and Dex on growth plate chondrocytes may result in a net increase in bioactive GC at the tissue level through alterations in GC pre-receptor metabolism [25]. Alterations in GC metabolism at the cellular level may impair the PI3K intracellular IGF-I pathway, and may be a common mechanism through which pro-inflammatory cytokines and GCs alter skeletal growth. Preventing the disruption of this pathway could potentially provide benefits in the treatment of pediatric chronic inflammatory diseases.

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CAPTIONS TO ILLUSTRATIONS

Figure 1: Effects of LY and PD (both 10μM) on ATDC5 proliferation in the presence/absence of A: Dex (10^{-6}) and B: IL-1β (10ng/ml). Mean + 1 S.E.M., n=6. $^{**}P<0.01$, $^{***}P<0.001$; NS = no significant difference.

Figure 2: Metatarsal growth in the presence of IGF-I over a 10-day period following treatment with A: LY and PD (both 10μM) and B: UO (10μM). Mean + 1 S.E.M., n=6; $^{***}P<0.001$ compared to IGF-I.

Figure 3: Metatarsal growth in the presence of IGF-I over a 12-day period following treatment with LY (10μM) in the presence of A: Dex (10^{-6}) and B: IL-1β (10ng/ml). Mean + 1 S.E.M., n=6; $^{***}P<0.001$ compared to IGF-I.